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### Title

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### Permalink

<https://escholarship.org/uc/item/9z181557>

### Journal

Nature biotechnology, 28(7)

### ISSN

1087-0156

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### Publication Date

2010-07-01

### DOI

10.1038/nbt.1645

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Author manuscript

*Nat Biotechnol.* Author manuscript; available in PMC 2011 January 01.

Published in final edited form as:

*Nat Biotechnol.* 2010 July ; 28(7): 738–742. doi:10.1038/nbt.1645.

## Chemical genetics of TOR identifies an SCF family E3 ubiquitin ligase inhibitor

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### Abstract

The target of rapamycin (TOR) plays a central role in eukaryotic cell growth control<sup>1</sup>. With prevalent hyper-activation of the mTOR pathway in human cancers<sup>2</sup>, novel strategies to enhance

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Methods are available as supplementary information.

Supplementary Information

Supplementary information accompanies this paper.

The library database and complete genomic datasets are available on the web (<http://labs.pharmacology.ucla.edu/huanglab>).

TOR pathway inhibition are highly desirable. We used a yeast-based platform to identify small-molecule enhancers of rapamycin (SMERs) and discovered an inhibitor of the SCF<sup>Met30</sup> ubiquitin ligase (SMER3). The large SCF (Skp1-Cullin-F-box) family of ubiquitin ligases performs important functions in diverse cellular processes including transcription, cell-cycle control, and immune response<sup>3</sup>. Accordingly, there would be great value in developing SCF ligase inhibitors that act by a defined mechanism to specifically inactivate ligase activity. We show here that SMER3 selectively inhibits SCF<sup>Met30</sup> *in vivo* and *in vitro*, but not the closely related SCF<sup>Cdc4</sup>. Our results demonstrate that there is no fundamental barrier to obtaining specific inhibitors to modulate function of individual SCF complexes, and suggest new strategies for combination therapy with rapamycin.

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Conserved from yeast to humans, the target of rapamycin (TOR) protein is a serine/threonine protein kinase that controls various aspects of cellular growth by regulating translation, transcription, autophagy, cytoskeletal organization, and metabolism<sup>1</sup>. Rapamycin, a secondary metabolite produced by *Streptomyces hygroscopicus*, specifically inhibits the activity of TOR resulting in starvation-like phenotypes. Over the past few years, deregulation of pathways upstream and downstream of mammalian TOR (mTOR) has been implicated in a variety of cancers, making the TOR signaling pathway a potential target for cancer therapy and rapamycin (and its analogs) an attractive anti-cancer agent<sup>2</sup>. Results from first round clinical trials suggest that different types of tumors have different sensitivities to rapamycin and in many cases rapamycin does not completely halt the progress of disease<sup>4, 5</sup>, thus making it desirable to identify small molecules that can act in concert with rapamycin. Although combination strategies taking advantage of known interacting pathways (*e.g.*, mTOR and IGF1R, PI3K or AKT) are being enthusiastically pursued<sup>6,8</sup>, an unbiased search for novel exploitable pathways has not been reported. The unbiased cell-based approach as described here has the potential to elucidate new interactions of TOR signaling with other pathways and to provide valuable chemical tools to study signaling networks in various settings.

We and others have previously shown that yeast is a promising platform for high-throughput discovery of small molecule modifiers of rapamycin-sensitive TOR functions, including both suppressors (SMIRs) and enhancers (SMERs), which show translatable potential for modulating TOR-related processes in higher organisms<sup>9, 10</sup>. Here we used the yeast-based screen to identify new SMERs targeting cell growth control (Supplementary Information). Using a ChemBridge DiverSet library containing 30,000 small molecules, we identified >400 compounds that, in the presence of a sub-optimal rapamycin concentration, gave a “no growth” phenotype (Supplementary Dataset 1). After gating out toxic compounds using unrelated screening datasets (Supplementary Information), a total of 86 potential SMERs were identified, which were synthetic sick/lethal with rapamycin but showed little toxicity by themselves at the concentrations used (Supplementary Dataset 2 and Supplementary Fig. 1). The SMERs encompass a variety of modes of action and biological activities, including direct inhibition of mTOR kinase activity, new post-translational regulation of mTOR function, and inhibition of patient-derived brain tumor initiating cells (manuscripts in preparation). Five structurally distinct molecules that exhibited differing effects on growth (Supplementary Information) were selected for further analysis (Fig. 1a).

The primary challenge for phenotype-based chemical genetic screens is the subsequent target identification, for which a variety of technologies—from affinity to genomics based—have been developed (see ref <sup>11</sup> and reviews therein). For our first efforts toward understanding SMER targets, we sought to take advantage of the tremendous information on gene expression related to various cellular pathways in yeast and performed genome-wide expression profiling using DNA microarrays. We hoped to link expression profile changes induced by SMERs with gene expression changes caused by genetic perturbations. This seemed an effective and elegant approach for target identification<sup>12</sup> and only requires that the potential drug target(s) be present in the cell. In order to capture early/direct transcriptome changes and avoid secondary effects, cells were treated with SMERs for a short period (30 min) and the extracted RNA was processed to probe Affymetrix GeneChips (Supplementary Information). Two classical ways of analyzing gene expression data are hierarchical clustering and principal components analysis (PCA). As shown in Fig. 1b, the clustering pattern of our microarray data classified the five SMERs identified from the screen into three distinct groups. Treatment of yeast cells with SMER2, 4 or 5 had no obvious effect on global gene transcription, whereas SMER1's effect on transcription shared extensive similarity with rapamycin (M.A. *et al.*, in preparation). SMER3's expression profile, on the other hand, is different from all the others. Consistent with hierarchical clustering, principal components analysis (Fig. 1c) also readily distinguishes these effects on gene expression.

We focused primarily on SMER3 given its distinct profile. Interestingly, a set of methionine biosynthesis genes (referred to as *MET*-genes hereafter) was upregulated in SMER3-treated cells (Supplementary Tables 1-2). GO analysis revealed that, in addition to the enrichment of sulfur metabolism genes among the induced group, genes involved in cell-cycle regulation were overrepresented in the downregulated group of SMER3-specific genes (Supplementary Tables 1-2).

Induction of *MET*-gene expression in response to SMER3 exposure suggested that the cellular pathway controlling homeostasis of sulfur-containing compounds was a possible target for SMER3. The key regulator of this pathway is the ubiquitin ligase SCF<sup>Met30</sup>, which restrains the transcriptional activator Met4 in an inactive state in methionine-replete medium by attachment of a regulatory ubiquitin chain<sup>13</sup>. Inactivation of SCF<sup>Met30</sup> prevents Met4 ubiquitination, permitting the formation of an active Met4-containing transcription complex that induces expression of the *MET*-genes and blocks cell proliferation. One hypothesis to explain the *MET*-gene activation and growth inhibition in SMER3-treated cells is that SMER3 inhibits SCF<sup>Met30</sup>. In agreement with this notion, Met4 ubiquitination was blocked in cells exposed to SMER3 (but not rapamycin) (Fig. 2a). Furthermore, genetic analyses have previously demonstrated that deubiquitinated Met4 mediates cell cycle arrest upon inactivation of SCF<sup>Met30</sup> (ref <sup>13</sup>), and deletion of *MET4* rescues lethality of *met30* (ref <sup>14</sup>). Notably, *met4* cells were also less susceptible to growth inhibition by SMER3 (but not rapamycin, exemplifying specificity) (Fig. 2b and Supplementary Fig. 2). These findings are consistent with SMER3 being an inhibitor of SCF<sup>Met30</sup>. However, the incomplete resistance of *met4* to SMER3 (Fig. 2b) suggests that SMER3 likely has additional targets other than SCF<sup>Met30</sup> and that cell growth inhibition by SMER3 is not solely due to SCF<sup>Met30</sup>.

inhibition. This is not uncommon as even Gleevec, which was originally believed to be a highly specific inhibitor of BCR-Abl, is now appreciated to exert its biological effects through protein kinases in addition to its intended target<sup>15</sup>.

SMER3 enhances rapamycin's effect and also inhibits SCF<sup>Met30</sup>, suggesting a connection between the TOR and SCF<sup>Met30</sup> pathways. To test whether SMER3 functions as an enhancer of rapamycin through inhibition of SCF<sup>Met30</sup> we asked if genetic inhibition of SCF<sup>Met30</sup> could mimic SMER3 in the synergistic effect with rapamycin. Indeed, hypomorphic alleles of the individual components of SCF<sup>Met30</sup> and its E2 ubiquitin conjugating enzyme, Cdc34, were hypersensitive to rapamycin (Fig. 2c). The synthetic lethality with rapamycin appears to arise largely from reduced SCF<sup>Met30</sup> activity because inhibition of Cdc4, which forms a related, essential SCF<sup>Cdc4</sup> ubiquitin ligase, only resulted in minor rapamycin hypersensitivity (Fig. 2c). Together these results suggest that SMER3 enhances rapamycin's growth inhibitory effect by inhibition of SCF<sup>Met30</sup>.

To test whether SMER3 can directly inhibit SCF ubiquitin ligases, we assayed ubiquitination of well-established SCF substrates by purified SCF complexes *in vitro*. Indeed, addition of SMER3 to the ligase reactions inhibited ubiquitination of Met4 by SCF<sup>Met30</sup> in a dose-dependent manner, whereas SMER1 had no effect (Supplementary Fig. 3). To assess specificity of SMER3 we also examined *in vitro* ubiquitination of Sic1 by the related WD-40 repeat containing SCF<sup>Cdc4</sup>. For direct comparison of SMER3 effects, activities of SCF<sup>Met30</sup> and SCF<sup>Cdc4</sup> were analyzed in a single reaction mix containing both ligase complexes and their substrates Met4 and Sic1 (Fig. 2d). Due to the faster kinetics of the SCF<sup>Cdc4</sup> catalyzed ubiquitination, the Sic1 reaction was probed at two incubation times: first at 5 min corresponding to the linear range for the SCF<sup>Cdc4</sup> reaction (at which time there was no Met4 ubiquitination by SCF<sup>Met30</sup>), then at 25 min corresponding to the linear range of the SCF<sup>Met30</sup> reaction. Consistent with the selective *in vivo* effect of SMER3 on SCF<sup>Met30</sup>, *in vitro* ubiquitination of Sic1 was unaffected by SMER3 (Fig. 2d and e). In some experiments with SCF<sup>Cdc4</sup>, a modest effect is seen on high MW conjugates (data not shown), but it is clear from the direct head-to-head comparison where both enzymes are in the same tube that there is a very large difference in sensitivity of the two ligase complexes towards SMER3.

To investigate the mechanisms of specificity in the inhibition of SCF<sup>Met30</sup> by SMER3, we examined the association of Met30 and the SCF core component Skp1. We found that Met30 was no longer bound to Skp1 in cells treated with SMER3 (Fig. 3a), suggesting that SMER3 prevents the assembly of SCF<sup>Met30</sup> or induces SCF complex dissociation (Supplementary Information). We next asked whether SMER3 affects the binding of other Skp1 interactors or acts specifically on SCF<sup>Met30</sup>. Skp1-bound proteins were purified from cells treated with SMER3 or DMSO solvent control and their relative abundance was determined using SILAC-based quantitative mass spectrometry. Among the eleven identified F-box proteins, only binding of Met30 to Skp1 was significantly inhibited by SMER3 (Fig. 3b). Skp1 and Met30 protein levels were not affected by SMER3, nor were the interactions of the SCF core components Cdc53 (cullin) and Hrt1 (RING component) with Skp1 (Supplementary Fig. 4 and Fig. 3b).

To further address the specificity of SMER3 for Met30 *in vivo*, we compared the cell cycle arrest phenotype induced by SMER3 to that of temperature-sensitive mutants of Met30, Cdc4, and the SCF components induced by non-permissive temperatures. As shown in Figure 3c, SMER3 induces a phenotype resembling that of genetic inhibition of Met30, while genetic inhibition of general SCF components or the specific F-box subunit Cdc4 gives a completely different elongated cell cycle arrest phenotype. Inhibition of any of the SCF core components simultaneously blocks SCF<sup>Met30</sup> and SCF<sup>Cdc4</sup>, yet the arrest phenotypes of SCF core mutants strongly resemble Cdc4 inhibition (Fig. 3c). This indicates that the *cdc4* cell cycle arrest morphology is “dominant” to that of *met30* and that inhibition by SMER3 is indeed specific for Met30 without affecting Cdc4 or SCF in general. Additionally, while SMER3-treated *cdc4* temperature-sensitive mutant cells have a phenotype at permissive temperatures resembling genetic inhibition of Met30, their phenotype changes to that resembling Cdc4 inhibition when shifted to non-permissive temperatures (Fig. 3c), further demonstrating that SMER3 has little effect on Cdc4 *in vivo*.

To test direct binding of SMER3, we employed differential scanning fluorimetry (DSF)<sup>16</sup> using purified Met30-Skp1 vs. Skp1 proteins (Met30 cannot be obtained in isolation without Skp1). As shown in Figure 3d, the addition of SMER3 altered the melting temperature of Met30-Skp1, but not that of Skp1 alone, indicating that SMER3 does indeed directly target the Met30-Skp1 complex. The simplest model to explain the biochemical specificities of SMER3 is that it binds directly to Met30 but not Skp1. Since drug binding often stabilizes a folded state, or conformation, of its protein target, leading to increased resistance to protease digestion (as assayed by DARTS, which stands for drug affinity responsive target stability<sup>11</sup>), we tested whether protease susceptibility of Met30 is altered by the presence of SMER3. Indeed, when yeast cell lysates were proteolysed with thermolysin or subtilisin, we observed SMER3-dependent protection of Met30 (Fig. 3e, f and Supplementary Fig. 5), but not Skp1. These experiments suggest that Met30 is the direct molecular target of SMER3, although we cannot exclude that SMER3 binding to Met30 may require Skp1.

Met30 contains at its N-terminus the F-box motif, which binds Skp1, and at the C-terminus the WD-40 repeats which serve as protein-protein interaction motifs for substrate binding<sup>17</sup>. We found that the Met30 F-box, but not the Cdc4 F-box, was protected to a similar extent as full-length Met30 by the presence of SMER3 in DARTS experiments (Supplementary Figs. 6 and 7). In contrast, SMER3 failed to protect the WD-40 repeat domain of Met30 (Supplementary Fig. 6 and Supplementary Information). These results suggest that SMER3 may recognize the F-box motif of Met30, yet further investigation is required to understand how SMER3 binds to Met30.

In this study, we demonstrated that SMER3 (i) specifically inhibits *in vitro* ubiquitination by recombinant reconstituted SCF<sup>Met30</sup> (Fig. 2d, e and Supplementary Fig. 3), (ii) selectively disassembles or prevents assembly of SCF<sup>Met30</sup> but not other SCF complexes *in vivo* (Fig. 3a, b, c), and (iii) directly binds to Met30 (or Met30-Skp1 complex), but not Skp1 alone (Fig. 3d and e). Together, these experiments suggest that SMER3 specifically inactivates SCF<sup>Met30</sup> by binding to Met30.

Historically designing specific inhibitors for SCFs has been considered highly challenging due to their common scaffolding subunits and similar enzymatic steps<sup>18,21</sup>, reminiscent of the obstacles faced with kinase inhibitors<sup>22</sup>. The unexpected biological specificities demonstrated by this first-generation hit provide encouraging examples for such potential and highlight the importance of unbiased cell-based approaches in drug discovery and in biological studies.

In conclusion, we identified several small molecule enhancers of rapamycin from a phenotype-based chemical genetic screen. Genomic, genetic, and biochemical analyses indicate that one of the SMERs (SMER3) inhibits an E3 ubiquitin ligase in yeast, SCF<sup>Met30</sup>, which coordinates nutritional responses with cell proliferation. Since increasing evidence suggests that ubiquitin E3 ligases are involved in tumorigenesis<sup>23</sup>, we believe that SMER3 and SMER3-like molecules represent a novel class of E3 ubiquitin ligase inhibitors that can potentially be used as anti-cancer drugs in the future.

In addition, our study provides the first link between the TOR pathway and a separate network that monitors the sulfur-containing amino acids methionine, cysteine and the primary methyl group donor SAM. This genetic interaction may be simply explained by the convergence of these two pathways on regulation of the G1 cyclins (refs <sup>14</sup>, <sup>24</sup>, and see Supplementary Table 2 for SMER3). Alternatively, it is possible that more complicated co-regulations occur in which TOR inhibition, while insufficient for activation of the “sulfur starvation” response, may in fact enhance this response during times of sulfur depletion (Supplementary Information). Although the target pathway for SMER3 in mammalian cells has yet to be elucidated, it is noteworthy that cancer cells and tumors are particularly dependent on metabolic networks linked to methionine<sup>25, 26</sup>, indicating that mammalian processes similar to that controlled by SCF<sup>Met30</sup> in yeast might provide potential anti-cancer targets. Alternatively, or additionally, it is possible that SMER3 may target  $\beta$ -TrCP (ref <sup>27</sup>) or related SCF(s) in humans whose genome contains many more F-box proteins. Synthetic lethal interactions between rapamycin and the ubiquitin-like modification systems (Fig. 2c) suggest potential therapeutic benefit for combination therapy with rapamycin and any small molecule that inhibits a component of SCF or an activator of SCF, such as in sensitizing a tumor's response to rapamycin and/or preempting the development of drug resistance. Beyond cancer and tumor-prone syndromes, a variety of other diseases including hypertrophy, neurodegeneration and aging are linked to the TOR pathway<sup>28, 29</sup>. For example, several SMERs have been identified which effectively enhance autophagy and reduce toxicity in Huntington's disease models through novel, so far unknown, mechanisms<sup>10</sup>. Similar chemical genetic approaches are applicable to the study of other pathways, drugs, and diseases.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

We are grateful for grant support from ACS and NIH, and for traineeship support of MA and BL by the NIH UCLA Chemistry–Biology Interface Predoctoral Training Program. NZ and RJD are investigators of the HHMI. We thank

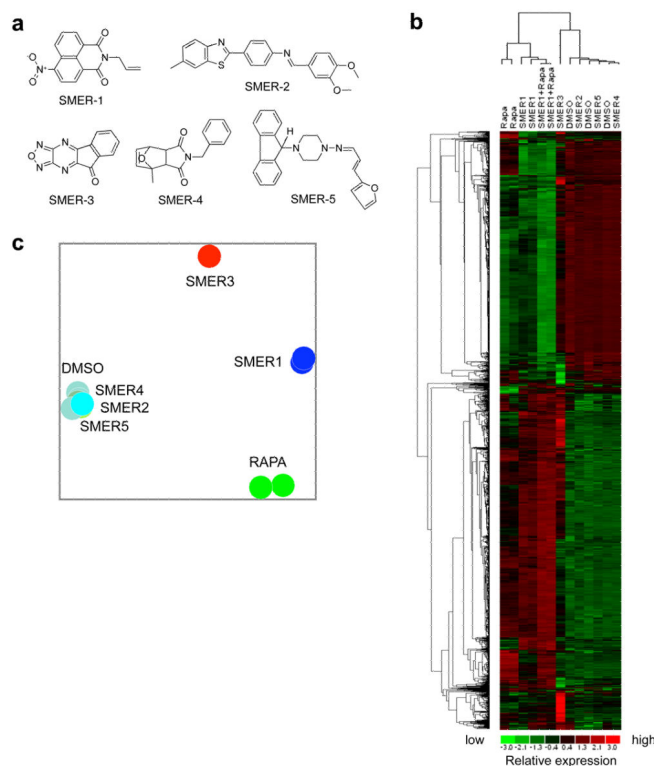
D. Skowrya (Saint Louis University) and M. Tyers (University of Edinburgh, UK) for their generous gifts of baculo virus constructs and anti-Met4 antibody, respectively. We also thank J. Salcedo (Roche Diagnostics Corporation) for support towards differential scanning fluorimetry experiments.

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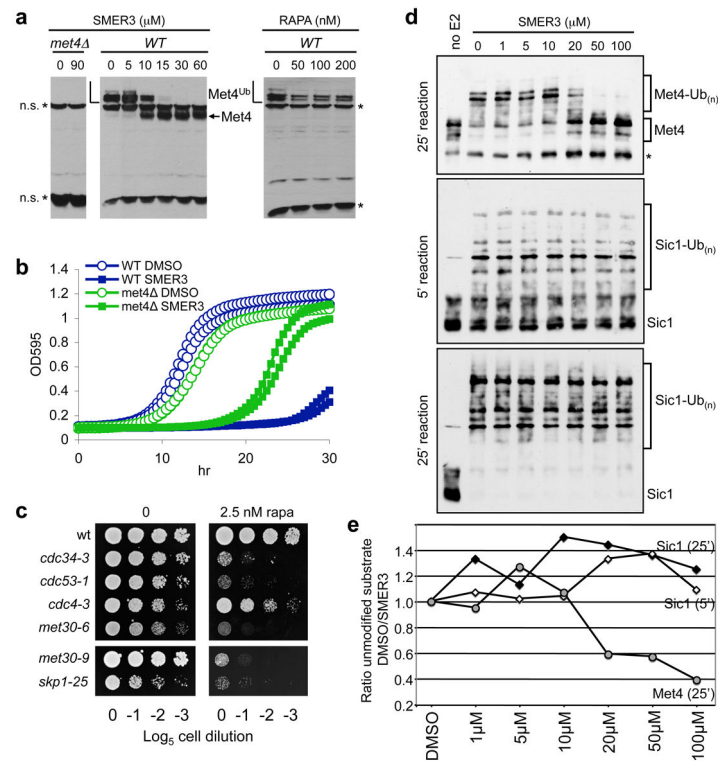


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**Figure 1. Two unsupervised data analyses classify five SMERs into three different groups based on their gene expression profiles**

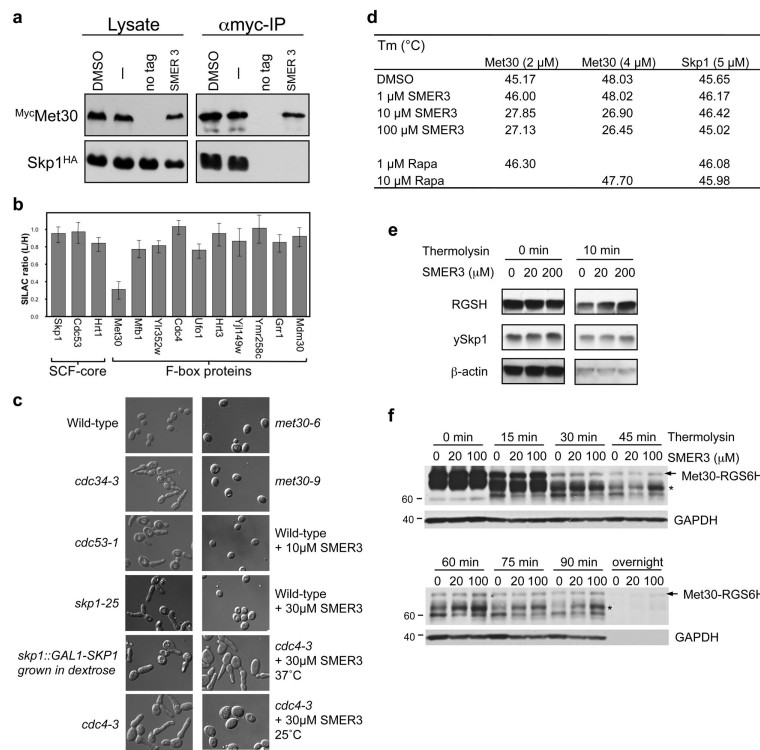
**a.** Chemical structures of SMER1 to SMER5. **b.** Two dimensional (2-D) hierarchical clustering reveals that the expression profile of SMER1 is similar to that of rapamycin, whereas the profiles of SMERs 2, 4 and 5 are indistinguishable from that of DMSO (solvent) control. The profile of SMER3 is distinct. Each row corresponds to a gene, and each column corresponds to an experimental sample. **c.** Principal component analysis is consistent with hierarchical clustering. Light blue: DMSO; blue: SMER1; cyan: SMER2; red: SMER3; sage: SMER4; chartreuse: SMER5; green: rapamycin. Replicates were obtained from independent small molecule treatments in separate experiments.



**Figure 2. SMER3 targets SCF<sup>Met30</sup>**

**a**, Biochemical evidence for SCF<sup>Met30</sup> inhibition by SMER3 but not rapamycin. Yeast cells were cultured in YPDA medium to mid-log  $0.8 \times 10^7$  cells/ml, treated with indicated concentrations of SMER3 or rapamycin for 45 min, and total protein was extracted for Western blot analyses (Supplementary Information). Met4 ubiquitination *in vivo* can be directly assessed by immunoblotting because ubiquitinated forms of Met4 are not subjected to proteasomal degradation and can thus be detected due to a characteristic mobility shift on denaturing gels<sup>30</sup>. Asterisk (\*) denotes a non-specific band immuno-reactive to the anti-Met4 antibody (generous gift from Mike Tyers). **b**, SMER3 resistance in *met4Δ* cells. Yeast cells were treated with either vehicle (DMSO) or 4 μM SMER3 and growth curve analysis was performed with an automated absorbance reader measuring O.D. at 595 nm every 30 min (Supplementary Information). Cell growth was measured in liquid because SMER3 activity is undetectable on agar. **c**, Genetic interaction between SCF<sup>Met30</sup> and TOR. Temperature sensitive mutants as indicated were grown at 25°C to mid-log phase in YPDA medium and serial dilutions were spotted onto plates with or without 2.5 nM rapamycin. The plates were incubated at the permissive temperatures for the mutants: 28°C for *cdc34-3*, *cdc53-1*, *cdc4-3* and *met30-6* because these mutants exhibited fitness defects at 30°C even without rapamycin, or 30°C (standard growth temperature) for *met30-9* and *skp1-25* because these alleles are not temperature sensitive until at 37°C. **d**, SMER3 specifically inhibits SCF<sup>Met30</sup> E3 ligase *in vitro*. Components of SCF<sup>Met30</sup> were co-expressed in insect cells and the complex was purified based on a GST-tag fused to Skp1. Met4 expressed in insect cells was bound to SCF<sup>Met30</sup> and the ligase-substrate complex eluted with glutathione. Purified ligase-substrate complexes were combined with purified SCF<sup>Cdc4</sup> and phosphorylated Sic1 and pre-incubated with DMSO or the indicated concentrations of SMER3 for 20 min at

room temperature. The ubiquitination reaction was initiated by addition of E1, E2, ubiquitin, and ATP. The reaction was allowed to proceed for 25 min, with an aliquot of the reaction collected after the first 5 min to accommodate different reaction kinetics by the two SCFs. Reaction products were analyzed by immunoblotting. The asterisks indicate a protein cross-reacting with the anti-Met4 antibody. **e**, The amount of un-ubiquitinated substrate (Met4 and Sic1) was quantified on a Fuji LAS-4000 imaging system and inhibition was expressed as the ratio of un-ubiquitinated substrate in DMSO/SMER3.



**Figure 3. Molecular mechanism for the specificity of SCF<sup>Met30</sup> inhibition by SMER3**

**a**, Protein-protein interaction between Met30 and Skp1 is diminished by SMER3 *in vivo*. Yeast strains expressing endogenous 13Myc-tagged Met30 were either untreated, or treated with solvent control (DMSO) or 30 $\mu$ M SMER3 for 30 minutes at 30°C. 13MycMet30 was immunopurified and immunocomplexes were analyzed for Skp1 binding by Western blot analysis. **b**, SMER3 specifically targets SCF<sup>Met30</sup> *in vivo* as determined by quantitative mass spectrometry. A yeast strain expressing endogenous HBTH-tagged Skp1 was grown in medium containing either heavy (<sup>13</sup>C/<sup>15</sup>N) or light (<sup>12</sup>C/<sup>14</sup>N) arginine and lysine to metabolically label proteins. The “heavy” culture was treated with solvent control (DMSO) and the “light” culture with 20 $\mu$ M SMER3 for 30 minutes at 30°C. Cells were incubated with 1% formaldehyde to cross-link and stabilize protein complexes *in vivo* for 10 minutes at 30°C. Cell lysates were prepared under denaturing conditions in 8M urea, mixed at equal amounts, and HBTHSkp1-bound complexes were sequentially purified on Ni<sup>2+</sup> and streptavidin sepharose under fully denaturing conditions. Tryptic peptides of the purified complexes were analyzed by LC-MS/MS. Relative abundance of proteins was determined by measuring the peptide peak intensities. Abundance ratios for SCF components identified by multiple quantifiable peptides are shown as SILAC ratios of “light” (SMER3-treated) over “heavy” (DMSO-treated) peptide intensities. **c**, SMER3 specificity for SCF<sup>Met30</sup> vs. SCF<sup>Cdc4</sup> as verified by cell cycle arrest morphology. Temperature sensitive mutants were shifted to 37°C for 4 hours. The Skp1 depletion phenotype was observed after repression of Skp1 expression in dextrose medium for 12 hours. SMER3 treatment of cells was for 6 hours. **d**, SMER3 directly binds to Met30-Skp1, but not Skp1 alone as determined by differential scanning fluorimetry (DSF). Met30 and Skp1 were either co-expressed or Skp1 was expressed alone in insect cells and the complex was purified based on a GST-tag fused

to Met30, while Skp1 was purified based on a His-tag fused to Skp1. Protein, drug and Sypro Orange dye were added to 384-well plates and melting curve fluorescent signal was detected using the LightCycler 480 System II (Roche). Melting temperatures ( $T_m$ ) were determined by the LightCycler 480 Protein Melt Analysis Tool. **e**, SMER3 protects endogenous Met30 from protease digestion. Yeast cells expressing Met30-RGS6H were lysed and digested with thermolysin in the presence of SMER3 vs. DMSO control, and extent of proteolysis was analyzed by immunoblotting. **f**, SMER3 protects recombinant Met30 from protease digestion. Met30 was PCR-subcloned into pcDNA3.1(-) (Invitrogen) and expressed using Promega TnT T7 Quick Coupled Transcription/Translation System. Thermolysin digestion was performed using translated lysate incubated with SMER3 or vehicle control, and stopped by adding EDTA pH 8.0. Samples were subjected to 4-12% NuPAGE gradient gel (Invitrogen) and Western blotted with anti-RGSH (Qiagen) and anti-GAPDH (Ambion) antibodies. The asterisks (\*) indicate the Met30 fragment that is protected by SMER3 from protease digestion.